# New Techniques and Applications in Lipid Analysis

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Chapter 7

# Supercritical Fluid Chromatography: A Shortcut in Lipid Analysis[1]

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## Introduction

Supercritical fluid chromatography (SFC) offers many advantages to the lipid analyst, particularly in the area of applied fat/oil technology, where the speed of analysis is critical. Commercial development of capillary SFC equipment in the early 1980s provided the analytical chemist with a unique tool that permitted chromatography of lipid moieties, up to and exceeding 1000 daltons in molecular weight, that could be detected and quantified using the universal flame ionization detector (FID). By programming the density of the mobile phase, the analyst could change the solvent power of the mobile phase, thereby effecting high-resolution separations, particularly between oligomeric species or members of a homologous series of compounds. SFC can permit very rapid analysis of many compounds and mixtures having the above characteristics and typically employs a mobile phase of supercritical carbon dioxide (SC–$CO_2$) at temperatures that minimize thermal degradation of temperature-sensitive compounds.

In the 1990s the use of SFC as an analytical technique became preferred for reasons not originally envisioned in the 1980s. With concerns about the reduction of solvent use in the analytical laboratory (1), SFC became very competitive with high-performance liquid chromatography (HPLC) for certain types of lipid analysis, since the technique reduces solvent usage relative to HPLC considerably. In addition, as has been noted by King (2) and Borch–Jensen (3), SFC reduces the need for derivatization of lipid moieties prior to their analysis, as well as extensive sample preparation prior to the determinative chromatographic step. This is particularly true for cases in which SFC can be combined with analytical-scale supercritical fluid extraction (SFE) in the on-line mode (4) to yield simplified or fractionated solventless extracts for chromatographic assay.

SFC has been characterized and compared with other analytical techniques for a wide variety of sample matrices (5,6). The utilization of packed and capillary columns for the analysis of complex lipid samples has been reviewed by Laakso (7). Advances

[1]Names are necessary to report factually on available data: however, the USDA neither guarantees nor warrants the standard of the product, and the use of a name by USDA implies no approval of the products to the exclusion of others that may also be suitable.

in column packings and supercritical fluid technology make SFC suitable even for the separation of polar lipid analytes (8). The advantages and flexibility of SFC for the characterization of lipid compounds has also been documented by others (9–11). The recent implementation of SFC-compatible evaporative light scattering detectors (ELSD) should increase the various modes of SFC even more for lipid analysis.

In this chapter, the uniqueness of capillary SFC as an analytical technique will be demonstrated as it has been applied in our research program. The chromatography in these studies was accomplished using primarily 50 to 100 μm i.d., 10 to 15-meter capillary columns. Specific chromatographic conditions—temperature, density or pressure programming characteristics, and column type—are given for each analysis problem as it is discussed in the text that follows. Many of the SFC analyses were accomplished on a SB-Octyl capillary column (15 m × 50 μm i.d.; 0.25 μm film thickness) (Dionex, Inc., Sunnyvale, CA), held isothermally at 120°C, using a density programming from 0.28 g/mL to 0.66 g/mL at 0.006 g/mL/min. This has proven to be a very facile program and set of conditions that is amenable to a wide variety of lipid analysis problems we have encountered. Although this is a relatively long program, for reasons that will be discussed shortly, the analysis time can be shortened by altering the density or temperature program once the retention pattern of the solutes has been established and compounds of interest identified for quantitation.

It should be noted that SFC is a natural technique for our laboratory, which is heavily involved in the exploitation of supercritical fluid technology for purposes of extraction, reaction, and fractionation. Hence, the philosophy of implementing SFC that follows is designed to address these analysis problems. Such research has included the analysis of lipid-containing mixtures extracted or fractionated using SC–$CO_2$; the deformulation of cosmetic products; detection and quantitation of trace lipid moieties; and, particularly in the last two years, the monitoring of reactions involving lipid reactants and products.

## *The Features of SFC*

Before proceeding with some examples that highlight the convenience and versatility of SFC, it is worth discussing some of the salient features that make SFC somewhat unique among the chromatographic techniques applicable to lipids. Mobile-phase programming is perhaps the most important variable in capillary SFC; choice of stationary phase for the separation of lipid-type solutes plays a secondary role in effecting the desired separation. Figure 1 illustrates a typical SFC density program and the resultant separation of minor lipid constituents (α-tocopherol and cholesterol) from the triglyceride profile constituting fish oil. It should be noted that this SFC separation was performed on a fish oil concentrate contained in a nutraceutical capsule. Sample preparation prior to SFC consisted of squeezing the oil out of the capsule and diluting it with *n*-hexane before injection onto the capillary SFC column.

A SB-Methyl column, 50 μm i.d., 15 m in length (Dionex Corporation, Sunnyvale, CA), using the density program described in the previous section was used to effect the separation of the lipid components. Such density programs are

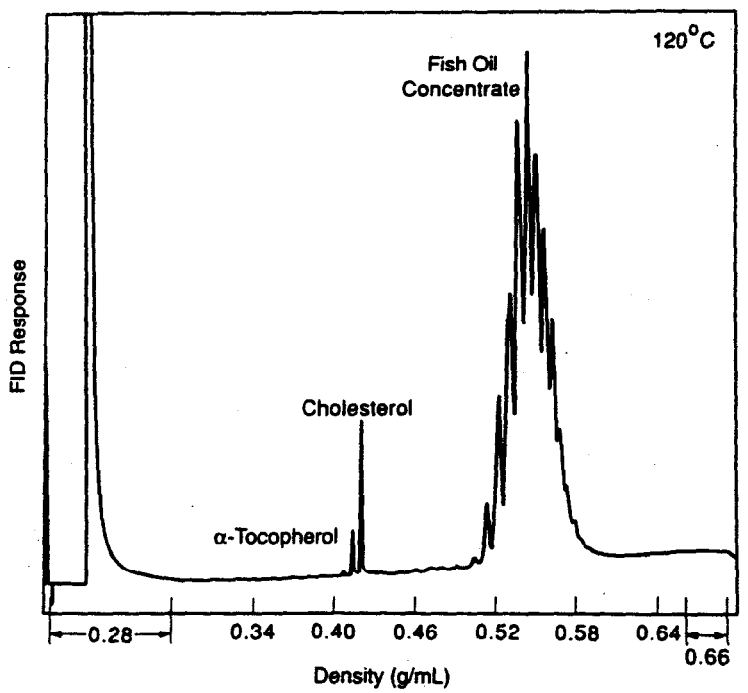


**FIG. 1.** Capillary SFC of fish oil from a nutraceutical capsule.

usually designed to provide a isoconfertic (constant-density) segment in the beginning of the program to allow the solvent peak to elute from the column. This is then followed by a ramping of the density or pressure as a function of time to a constant density or pressure, where the density or pressure is then held to ensure elution of the last solutes off of the column. The density program described (which is also reproduced on the horizontal axis of the chromatogram in Fig. 1) is fairly long (approximately 90 min), but such time-extensive programs are essential to understanding the molecular complexity of the sample being chromatographed. Once this is established, the programs may be further truncated with respect to time, or temperature programming may be brought into play, to shorten the analysis time or improve the resolution between the chromatographic peaks. One feature of the chromatographic separation illustrated in Fig. 1 is that the higher-molecular-weight components, which were of no interest in this assay (i.e., the fish oil triglycerides), can be density-programmed out of the column after the target analytes, cholesterol and α-tocopherol, have eluted. This eliminates further sample preparation or cleanup on the front end of this assay.

To illustrate the versatility provided by SFC mobile-phase programming, Fig. 2 shows the capillary SFC separation of a lipophilic additive, *p,p′* = DOPA (i.e., *p,p′* = dioctyldiphenylamine) from the main constituents in a lubricant designed for use in a space communication satellite. The major component in this formulation is Apiezon

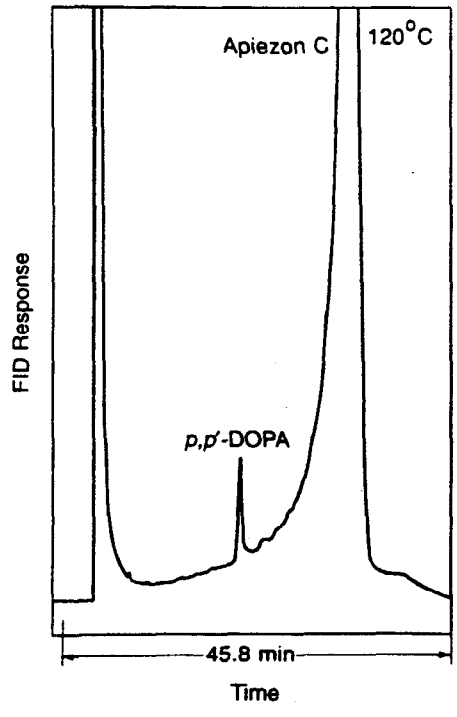


**FIG. 2.** Capillary SFC of a lubricant formulation for a space communications satellite.

C, which is a vacuum-distilled fraction consisting of a mixture of high-molecular-weight hydrocarbons. This separation was achieved without any prior sample preparation by dissolving the lubricant formulation in hexane, followed by a specially designed inverse asymptotic density program (12,13) to separate the amine additive from the Apiezon C mixture in only 45 min. The density program started at 0.35 g/mL and finished at 0.66 g/mL using an analysis temperature of 120°C. The SB-Methyl column described in the previous example was used in this assay. The described separation could not have been achieved in this time frame without resorting to SFC.

There is no doubt that the ability of combining the FID with SFC offers the analyst a unique universal sensitivity to most lipid compounds, similar to that experienced when using gas chromatography (GC)–FID, but with the additional advantage of having the ability to access higher molecular-weight compounds. In the early development of capillary SFC, it was often felt that quantitation of solutes primarily suffered because of the poor injection reproducibility the technique afforded. However, we have found for most of our samples that we can quantitatively reproduce our capillary SFC analyses without having to resort to more elaborate injection protocols described in the literature (14). We have also observed good linearity for the FID when used in conjunction with capillary SFC. Figure 3 illustrates the resultant calibration curve obtained from injecting millimolar (mM) quantities of the ester, octyl laurate, onto a SB-Octyl column. One can see that the calibration curve is very linear and that peak area can be used to calibrate the detector in terms of mM

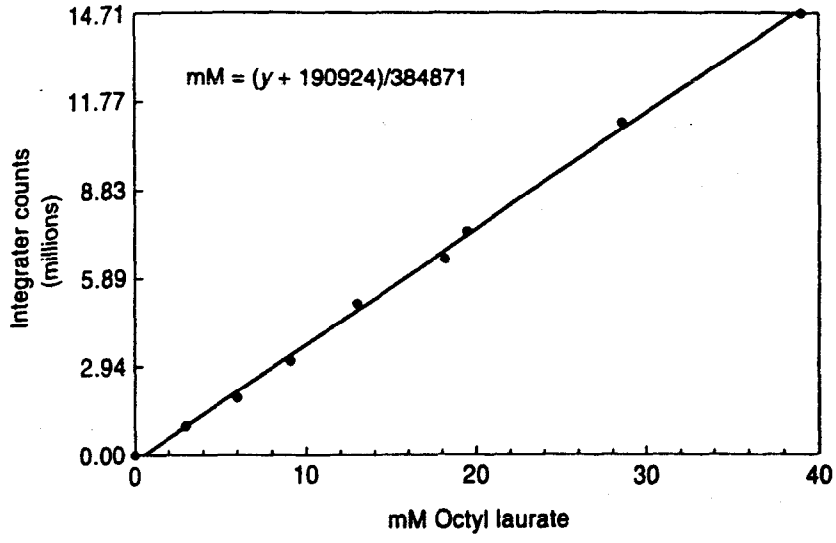


FIG. 3. FID calibration curve for octyl laurate synthesized by lipase catalysis in the presence of SC–$CO_2$.

response to octyl laurate. It is worth noting that the octyl laurate described above is an ester synthesized by lipase catalysis in the presence of flowing SC–$CO_2$, which is used to solvate and transport the acid and alcohol reactants over the catalyst bed (i.e., a supercritical fluid reaction).

## Retention Trends in Capillary SFC

Understanding the retention trends of solutes in capillary SFC is important, since such data plays a key role in the identification of lipid-type compounds and optimization of the separation pattern. To a first approximation, solutes undergoing pressure or density programming in capillary SFC elute according to increasing molecular weight; that is, higher molecular-weight components elute later than their lower-molecular-weight homologs (15,16). This principle is adequately illustrated in Fig. 4, where a mixture of linear, lipophilic alcohols are separated according to chain length with increasing density of the mobile phase. The aforementioned SB-Octyl column was used to effect this separation along with the density program previously described. The high resolution of the capillary column also allows isomers of the long-chain alcohols to be ascertained. Molecular weight determination of such homologs is possible by constructing plots of elution density vs. solute molecular weight (16).

The same elution principle applies in the case of coconut oil triglycerides pictured in Fig. 5. Since coconut oil is primarily composed of saturated triglycerides ranging from $C_{18}$ to $C_{54}$, density-programmed SFC of the oil yields an attractive chromatogram with equally spaced triglyceride peaks. This idealized

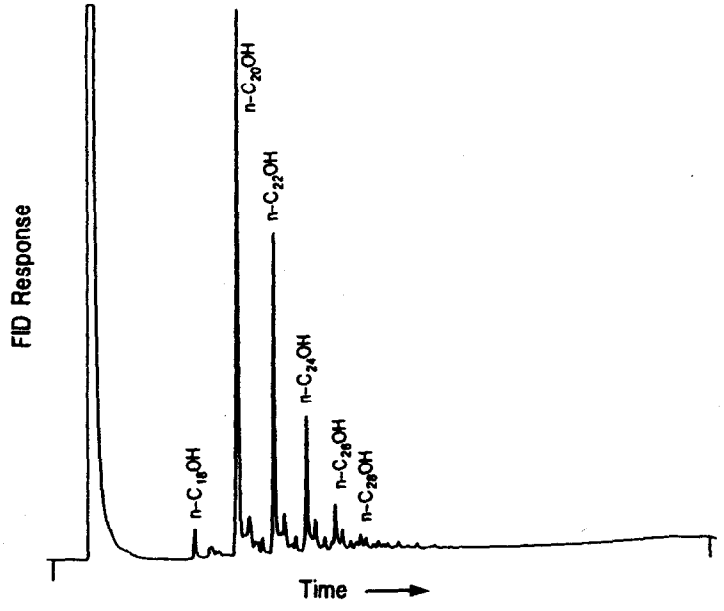


FIG. 4. Capillary SFC separation of a linear alcohol mixture.

separation was accomplished using the SB-Octyl column and the previously described density program from 0.28 to 0.66 g/mL. Such standard profiles for a variety of industrially used oils can be used as a relatively fast method for quality control. It should be noted that since many vegetable oils consist of mixed saturated and unsaturated triglyceride moieties, peak overlap is inevitable, making quantitation of the individual triglycerides difficult. The situation can be improved

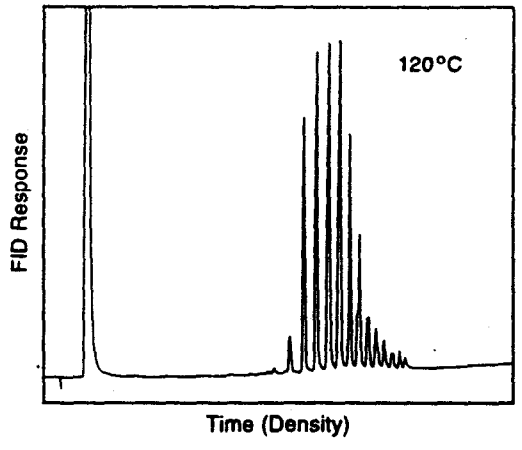


FIG. 5. Capillary SFC of a coconut oil sample.

somewhat by employing inverse temperature programming or a change in the column stationary phase (17).

The elution pattern for mixed lipid species in capillary SFC using nonpolar columns has been characterized by Borch-Jensen (3) and is given in Fig. 6. Once again, the overall retention pattern for a variety of lipid solutes is molecular weight-dependent; however, as can be seen in Fig. 6, there is adequate resolution between the different lipid groups, to permit useful separations and quantitation to be attained. In general, fatty acids elute first from nonpolar capillary SFC stationary phases, because of their lower molecular weights and higher polarity; they are followed by hydrocarbonaceous solutes, such as squalane, squalene, and the fat-soluble vitamin group, and then sterols, such as cholesterol. The higher molecular-weight wax esters occupy an intermediate elution position and can overlap with diglyceride moieties if both are present in a sample matrix. Again, depending on the molecular weights of the individual species and their presence in a sample matrix, one finds that cholesteryl esters, ether-containing lipids, and triglycerides tend to emerge late in the elution profile. It is has been our experience that many of the common cholesteryl esters elute adequately before major triglycerides found in animal- and vegetable-derived oils. Thus, Fig. 6 should be regarded as the worst possible scenario for the capillary SFC of complex lipid mixtures, since most lipid-containing mixtures will contain only some of the individual species within the groups shown in Fig. 6. It should be noted that fatty acid methyl esters (FAMEs), a commonly encountered lipid species, usually elute before the corresponding free fatty acid moieties, based on vapor pressure considerations.

An excellent example of the capillary SFC of a complex lipid mixture is shown in Fig. 7 (18), where the components present in deodorizer distillate have been nicely

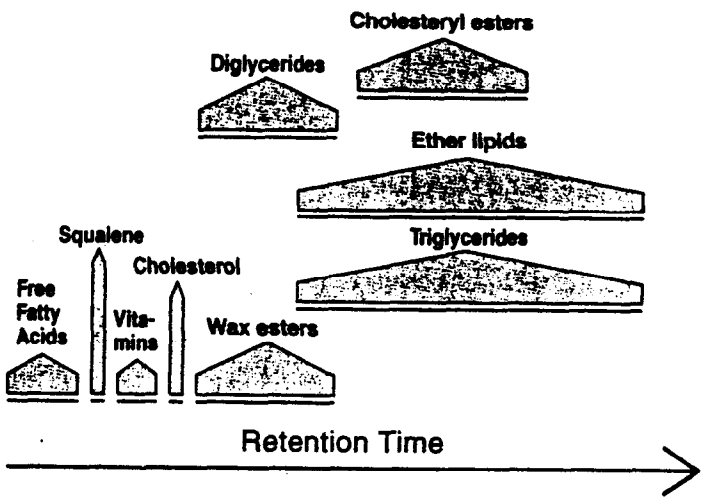


FIG. 6. Retention pattern for lipid classes and solutes for capillary SFC on nonpolar stationary phases (Ref. 3).

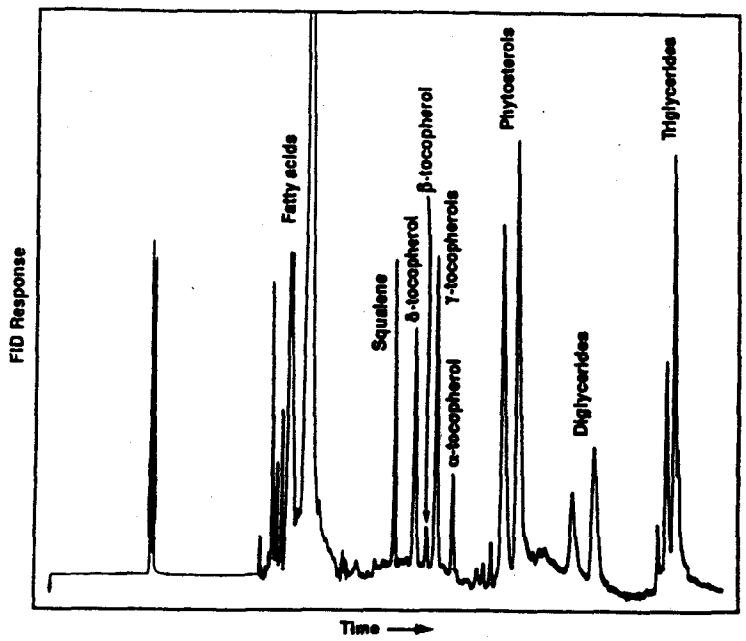


FIG. 7. Separation of lipid components in deodorizer distillate by capillary SFC.

separated using a SB-Octyl-50 column employing both pressure and temperature programming simultaneously. One can see that the retention pattern described by Borch–Jensen holds: fatty acids followed by squalene, the tocopherols (vitamin E precursors), sterols, diglycerides, and finally triglycerides. For the pressure program, the initial pressure was set at 100 atm for 10 min and then was increased at a rate of 5 atm/min to 180 atm. At that point the ramp rate was changed to 2 atm/min until a pressure of 220 atm was reached. Then the pressure increase rate was changed to 5 atm/min until a pressure of 350 atm was attained. The corresponding temperature program as a function of time consisted of holding at 100°C for 10 min, then increasing the temperature to 180°C at a rate of 5°C/min, followed by decreasing the oven temperature from 180 to 100°C at a rate of −5°C/min. This is a excellent example of how the versatility of SFC programming allows a separation pattern to be optimized.

The authors know of no other chromatographic technique that can perform such a separation encompassing so many different lipid species over such a large molecular weight range. Obviously, such methods could be used for the assay of tocopherol antioxidant mixtures as well as vitamins.

## SFC in Comparison with Other Techniques

There are few comparisons in the literature where results derived from SFC are compared to those determined by other chromatographic or chemical analysis

methods. A particularly thorough study has been conducted by Lee, Bobik, and Malone (19) for the determination of mono- and diglycerides in commercial emulsifier samples, using SFC on both derivatized and underivatized samples and comparing these results with data derived from a GC-derivatization method. The agreement between methods was found to be very satisfactory, and relative standard deviations for the various methods varied between 1.88 and 3.98%. Similarly, we have used capillary SFC for the analysis of the diglyceride content in a randomized lard sample developed for use in a feeding study. These results are presented in Table 1, where they are compared to those derived from GC–FID analysis, three HPLC-based methods, and a TLC assay of the randomized lard sample. It is interesting to note that there is considerable variation between the six listed methods with respect to the sample's diglyceride content; hence SFC is no better or worse then any of the other listed lipid analysis techniques. Results for the major component in the sample, the triglycerides, agree much better from one method to the other. It is worth noting that capillary SFC was competitive with the other listed methods with respect to analysis time; the SFC assay took only 25 min, whereas GC–FID, HPLC–ELSD, and TLC methods required 30 min for completion.

We have also used capillary SFC to determine the mixed tocopherol content in a deodorizer distillate sample and a commercial antioxidant formulation using the SFC method previously described for the deodorizer distillate matrix. Table 2 compares the results that we obtained with those derived from a GC method that required derivatization of the tocopherols to effect chromatography. There are some differences between the results derived by capillary SFC and the GC-derivatization method; in general, SFC yields slightly higher results then those found via the GC method. This could reflect the loss of analyte in the derivatization step of the GC method. Both chromatographic methods yielded similar relative standard deviations, indicating that the precision experienced using both methods is nearly identical. Although there is no inherent reason to expect SFC to be any less precise or accurate

**TABLE 1**
Analysis of Glyceride Content of a Randomized Lard Sample by Different Chromatographic Methods

| Analysis Method | %MG[a] | %DG[a] | %TG[a] | Time |
|---|---|---|---|---|
| SFC–FID[b] | 0.2 | 9.6 | 90.1 | 25 min |
| GC–FID[c] | 0.1 | 6.9 | 92.9 | 30 min |
| HPLC–FID[d] | | 14.5 | 86.5 | 1 h |
| HPLC–ELSD | | 8.0 | 92.0 | 30 min |
| Silica column[e] | 1.0 | 7.7 | 91.3 | 2 h |
| TLC[f] | 2.0 | 11.0 | 87.0 | 30 min |

[a]MG = monoglycerides; DG = diglycerides; TG = triglycerides.

[b]SFC method used for SFE/SFR.

[c]GC analysis accomplished by a high-temperature column.

[d]HPLC column.

[e]Silica column.

**TABLE 2**
Comparison of Methods for Determination of Tocopherol in Deodorizer Distillate and Commercial Antioxidant Samples

| | Deodorizer distillate | | Antioxidant | |
|---|---|---|---|---|
| Type of tocopherol | SFC[a] | GC[a] | SFC[a] | GC[a] |
| α-tocopherol | 2.37 (6.95)[b] | 1.59 (3.62) | 7.21 (2.04) | 8.98 (1.54) |
| β-tocopherol | 0.58 (7.55) | 0.36 (10.89) | 0.52 (20.39) | 0.24 (21.15) |
| γ-tocopherol | 8.56 (4.20) | 7.14 (3.32) | 23.47 (2.49) | 22.39 (1.46) |
| δ-tocopherol | 6.24 (2.68) | 4.89 (5.62) | 25.24 (1.79) | 24.49 (1.90) |

[a]Average of five analyses: values in mg/100 mg.
[b]Numbers in parentheses are relative standard deviations.

then any other analytical technique, it suffices to say that more studies of this type need to be carried out in the future.

## Applications of Capillary SFC to Lipid-Containing Samples

### *Monitoring SFE and SFF (Supercritical Fluid Fractionation) Processes*

As noted previously, SFC is natural analysis tool for monitoring the results obtained from SFE and SFF experiments. The rationale behind this statement is that if solutes can be solubilized in supercritical fluids (particularly SC–$CO_2$), then they probably are amenable to chromatography using the same supercritical fluid media. Only in the case of extractions or fractionations carried out at pressures or temperatures beyond the instrumental capabilities of analytical SFC should a degree of caution be exercised. We have primarily used capillary SFC to analyze extracts and fractions after their collection from the process under study (i.e., off-line analysis) as opposed to integrating the SFC for on-line, real-time analysis. Several examples of this type of SFC analyses follow.

We have extracted evening primrose oil from the seeds of *Oenothera biennis* L., since the oil is a source of γ-linolenic acid, a fatty acid entity having reported therapeutic value (20). Whereas it is well known that the fractionation of lipid moieties can be effected by changing the pressure and temperature at which process SFE is conducted, we were also interested in monitoring whether fractionation of the oil's triglycerides was occurring during the course of the SFE conducted at a constant pressure and temperature. Table 3 shows the triglyceride composition of the collected oil according to carbon number from both SFE and conventional Soxhlet extraction as determined by capillary SFC. From this data it is apparent that the SFE conducted at 70 MPa and 50°C yields an equivalent extract in terms of the triglyceride composition as determined by SFC. However, capillary SFC analysis shows that this is not the case for the two extracts obtained at 20 MPa and 40°C. Here a time-based fractionation effect is apparent, since the triglyceride composition has changed between the first and last fractions (particularly for the $C_{52}$ and $C_{56}$ triglycerides). It is interesting to note that GC analysis of fatty acid methyl esters (FAMEs)

**TABLE 3**
Normalized Triglyceride Composition of Evening Primrose Oil Extracts as Determined by Capillary Supercritical Fluid Chromatography

| Extraction type | % Triglycerides | | | |
|---|---|---|---|---|
| | $C_{50}$ | $C_{52}$ | $C_{54}$ | $C_{56}$ |
| Soxhlet (hexane) | 1.23 | 16.95 | 74.89 | 6.93 |
| SC–$CO_2$ at 70 MPa and 50°C (first fraction) | 1.27 | 16.92 | 75.01 | 6.80 |
| SC–$CO_2$ at 20 MPa and 40°C (first fraction) | 1.55 | 18.83 | 74.80 | 4.82 |
| SC–$CO_2$ at 20 MPa and 40°C (last fraction) | 0.67 | 13.04 | 75.20 | 11.09 |

on the above fractions did not show any disparity between the fatty acid compositions of the collected fractions. This indicates that SFC analysis provides somewhat different information on the fractions obtained from the SFE experiment.

Chromatographic profiling of an extract composition by capillary SFC can also provide valuable information as to how the SFE process is affecting the resultant extracts. As shown in Fig. 8, significant changes occur in the capillary SFC profile of a wool grease extract obtained via extraction with SC–$CO_2$ at 520 bar (52 MPa) and 80°C (21). The individual profiles in Fig. 8 represent in descending order extracts collected under these conditions at 210, 215, 217, and 290 min, respectively. It is apparent that as the extraction of the wool grease proceeds, the cholesterol content of the extract decreases, while the unresolved cluster of peaks, representing the wax ester content of the wool grease, also changes as a function of processing time. These changes manifest themselves in altering the melting point of the individual fractions (the melting point difference between the first and last fractions shown in Fig. 8 is approximately 17°C). These graphic SFC profiles were obtained using the aforementioned SB-Octyl column along with the density program previously described, which goes from 0.28 g/mL to 0.66 g/mL. The observed trends were also confirmed by thin-layer chromatography (TLC).

The monitoring of monoglyceride enrichment via a SFF process by capillary SFC has also been utilized in our laboratory (22). The objective of the SFF process was to produce a top product that could be used as an industrial emulsifier. A thermal-gradient fractionating column incorporating a flowing SC–$CO_2$ phase was utilized to enrich the monoglyceride content. Fractionation conditions were as follows: pressure of 31 MPa (4500 psi); temperatures in four consecutive zones from the bottom to top of the column were 65, 75, 85, and 95°C, respectively; $CO_2$ flow rate of 10 L/min (expanded $CO_2$ flow) with a feed flow rate of 1.2 mL/min. The monoglyceride content was increased about 20 wt. %, yielding a mixture equivalent to a commercially sold product. As shown in Fig. 9, SFC provided a total quantitative profile of the resultant top product composition that can be used to assess the results from the column fractionation process. Again, an SB-Octyl-50 capillary column (10 m × 100 μm i.d.; 0.5 μm film thickness) at 100°C was used, along with a pressure program

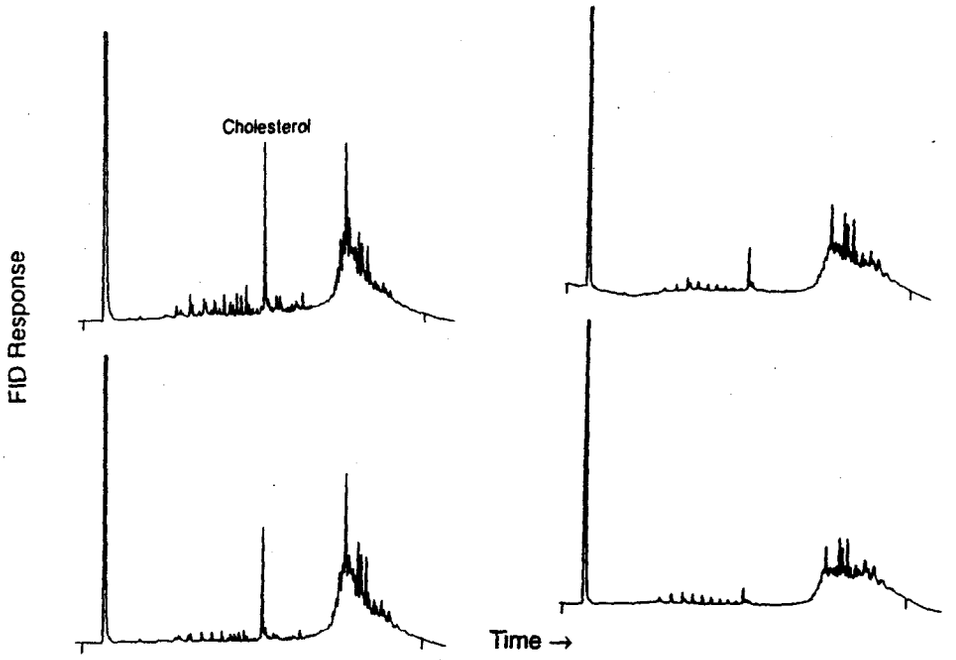


FIG. 8. SFC profiles of collected wool grease fractions obtained from SFE experiment.

from 100 atm to 300 atm at 8 atm/min (10) to effect the separation. The resultant profile is a good example of how the sequence noted by Borch–Jensen (3) can be utilized to characterize this mixed glyceride composition. The SFC profile also detects the presence of free fatty acids (FFA) and indicates that the feed material may contain (or the fractionation process may produce) the hydrolysis product (FFA) as a side product, rather than the desired mixed glyceride compositions.

### *Analysis of Minor Constituents in Lipid Mixtures*

SFC–FID can also be used for the detection and quantitation of trace components that frequently occur in lipid-rich matrices, provided the flame ionization detector has sufficient sensitivity for the target analyte. Analysts who routinely employ capillary SFC occasionally forget that the FID has a large dynamic detection range, and this potential is frequently suppressed when one is exploiting capillary SFC for the analysis of major components in lipid-containing mixtures. A good example of this principle is shown in Figs. 10 and 11, where capillary SFC has been performed on a peanut oil sample using the standard SB-Octyl column analysis described earlier in this chapter.

At the detector signal amplification levels normally used for the FID when capillary SFC is being used for triglyceride analysis (Fig. 10), the trace components in the peanut oil are hardly detectable, relative to the main triglyceride profile.

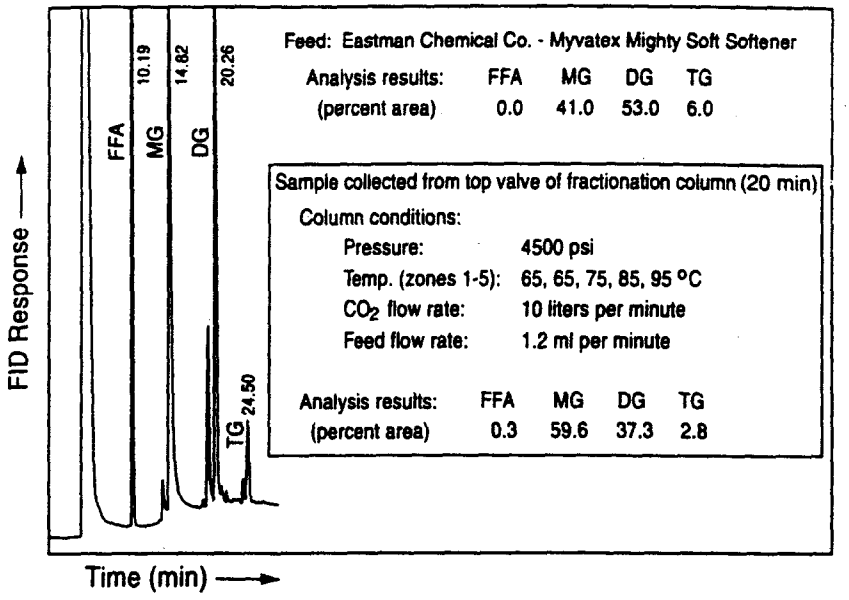


**FIG. 9.** Capillary SFC separation of glyceride mixture obtained from a SFF column experiment.

However, by increasing the sensitivity of the FID (Fig. 11), trace species become apparent in the chromatographic profile. For the peanut oil sample under consideration, there appears to be a series of peaks, perhaps associated with a homologous or oligomeric series of compounds, between the large solvent peak and the enlarged triglyceride profile. Mass spectrometric identification of these compounds indicated that they were a series of polysiloxane oligomers. This indicated that the peanut oil had been treated with agents to suppress its volatility and foaming as well as to suppress its deterioration via oxidation.

Another example of using capillary SFC for trace component analysis is to assess the cholesterol content of foodstuffs. SFC methods have been used for the analysis of cholesterol and cholesteryl esters in milk fat (23), egg (24), and human serum (24,25) and yielded accurate determinations. Our approach was to use analytical SFE in the off-line mode in a two-step sequence, in which the interfering lipid moieties (i.e., triglycerides) were initially removed from the sample matrix using SC–$CO_2$ an extraction cell packed with various sorbent media that would adsorb the cholesterol selectively. Then, after the removal of most of the interfering fat, we used the same extraction conditions, except for a small quantity of organic solvent added to the SC–$CO_2$ as a cosolvent, to effect removal of the cholesterol from the sorbent surface. To test the effectiveness of this scheme, we monitored the SC–$CO_2$ extracts both before and after the addition of cosolvent to the SC–$CO_2$ (Figs. 12A and B) using capillary SFC. The pressure program and column temperature for the analysis

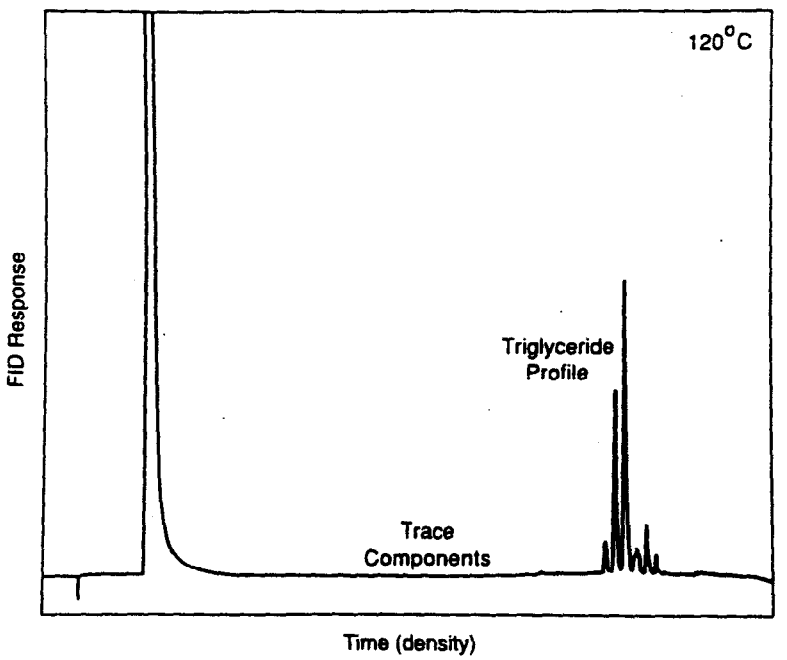


FIG. 10. Capillary SFC of a peanut oil sample using a low amplification of the FID signal.

are noted on Fig. 12. An SB-Octyl-50 column (100 μm i.d., 10 m in length, 0.5-μm film thickness) was used to effect the separation shown in Fig. 12.

Figure 12A shows that the cholesterol was effectively retained on the sorbent surface during SFE of the sample (the sample was the same fish oil from the capsule cited in Fig. 1). The large unresolved band in Fig. 12A is part of the triglyceride content contained in the fish oil sample. Figure 12B shows that addition of a cosolvent (methanol) to the SC–$CO_2$ released the cholesterol from the matrix, along with some of the excess fat also adsorbed on the sorbent surface. Note that the preliminary sample cleanup step, termed "inverse SFE" by the authors, has reduced the interfering fat band substantially—so much so that the interfering lipid species can be programmed out of the capillary SFC column after elution of the cholesterol. Had the fat level in the sample matrix not been reduced beforehand, it would probably have interfered with the cholesterol peak shown in Fig. 12B.

As shown in Fig. 13, the calibration linearity of the FID for cholesterol in this SFC application was excellent. Using the method just described, we have been able to recover cholesterol from a variety of fat-containing food matrices at recovery levels exceeding 75%. This provides, then, a technique that uses very low levels of organic solvent in the analytical laboratory on both the extraction and chromatography steps and can be used for cholesterol determination as mandated by the Nutritional Labeling and Education Act (NLEA).

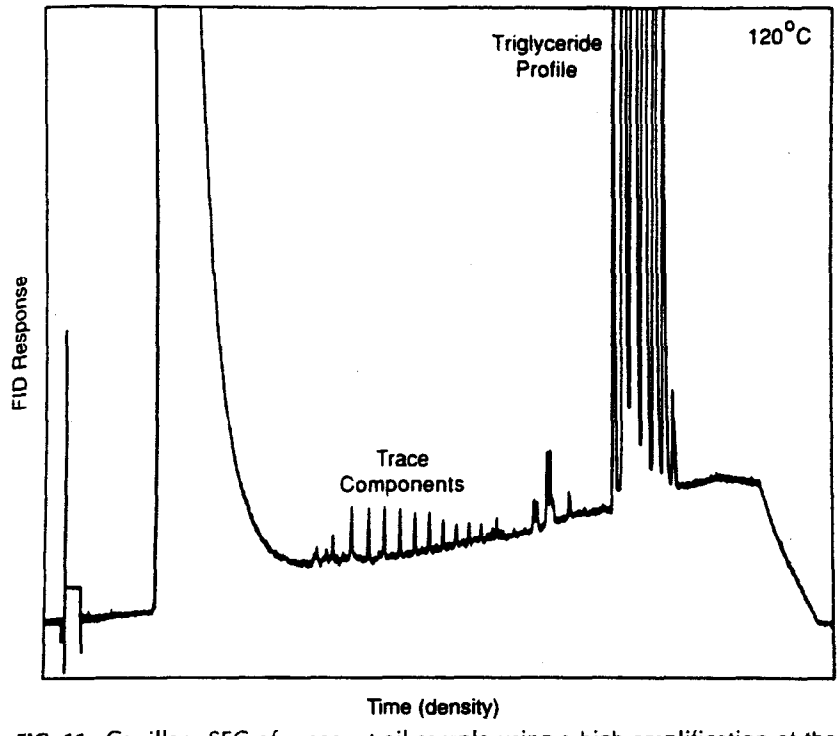


**FIG. 11.** Capillary SFC of a peanut oil sample using a high amplification of the FID signal.

### *Deformulation of Commercial Products*

We have found in our laboratory that capillary SFC is an excellent technique for deformulating complex commercial products because of the mobile-phase programming capabilities inherent in it and hence its ability to chromatograph a wide molecular weight range of nonpolar to moderately polar compounds. Control of the mobile-phase pressure or density with respect to time is very precise in commercial capillary SFC instrumentation, allowing a repeatability of retention time that is very precise. This feature of capillary SFC can be used in conjunction with analytical standards to identify many of the components that occur in commercial products that contain lipophilic ingredients.

An example of this precision in retention and how it can be utilized is shown in Fig. 14, where a supposed lanolin sample has been chromatographed by SFC. The density program utilized was the standard one developed for use with an SB-Octyl-50 column and mentioned earlier in this chapter. However, upon chromatographing the lanolin sample, it was found that the classic signature compound in lanolin samples, cholesterol, did not have the same retention time as provided by the injection of a cholesterol standard. However, the isolated peak that appears in Fig. 14 did

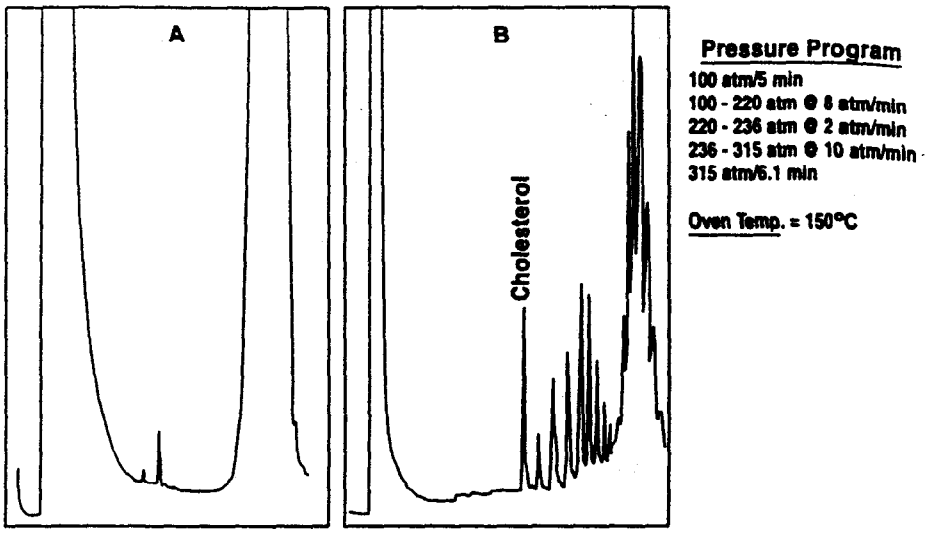


**FIG. 12.** SFC chromatograms of two fractions collected from an analytical SFE experiment to determine the cholesterol content in a fish oil capsule.

match the retention time of the cholesterol ester, cholesteryl acetate. This indicated that the sample we were chromatographing was not lanolin but acetylated lanolin, a derivative of lanolin utilized in cosmetic preparations because of its superior penetrating properties.

We have also used capillary SFC to deformulate certain types of cosmetic products, such as lipsticks and lip balms, and certain health aid products (4). In this case, approximately a 1% by weight solution of the product is dissolved in *n*-hexane and

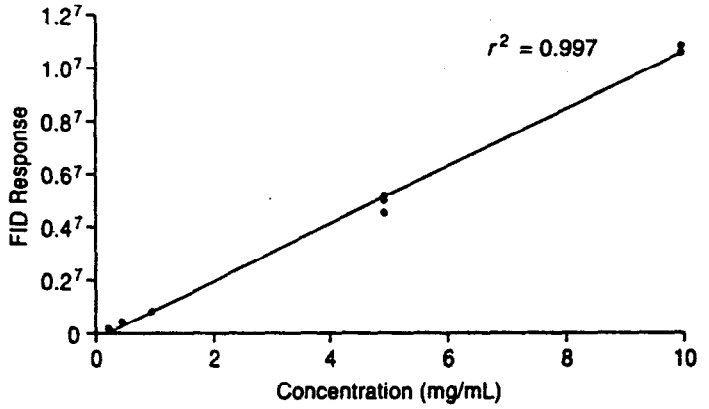


**FIG. 13.** Calibration curve for FID response to cholesterol using capillary SFC for the analysis.

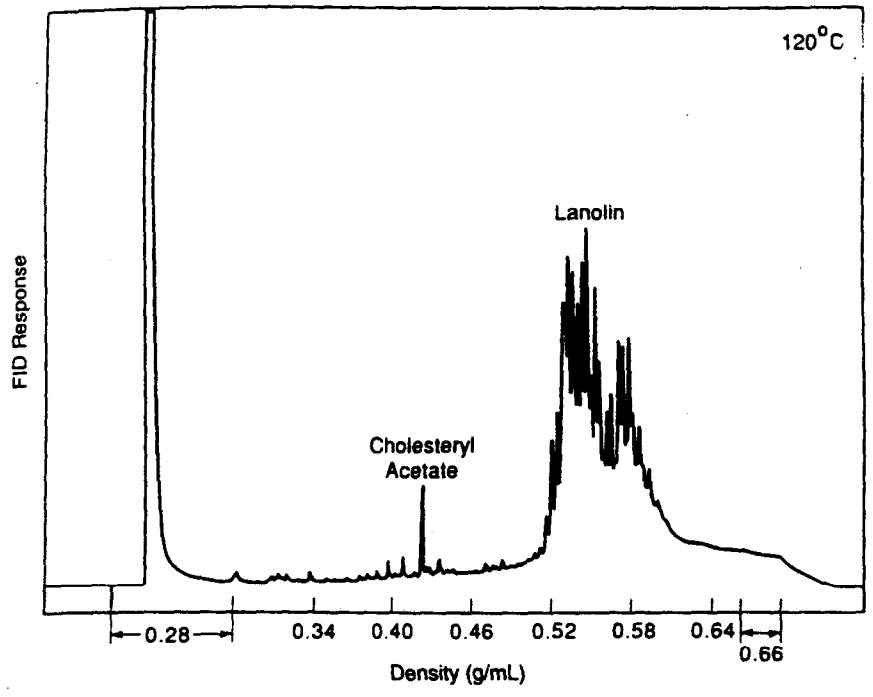


FIG. 14. Capillary SFC of acetylated lanolin sample.

the solution filtered if turbidity persists. An injection of this preparation is then made into the capillary SFC instrument, and then a lengthy density or pressure program is used to separate the dissolved components. Ingredients in the cosmetic formulation are identified by matching their retention times with known compounds or mixtures described in the formulation. In some cases, the ingredients have distinct patterns under SFC programming conditions (e.g., lanolin, petrolatum, or waxes), which aid in their identification. Using this approach, distinctly different chromatographic profiles are produced by different commercial products, thus aiding a formulator in matching a competitor's product.

An example of such a profile generated by the SFC method is shown in Fig. 15 for a decongestant ointment. Note that two volatile medicinal ingredients, camphor and menthol, elute at a mobile-phase density of approximately 0.20 g/mL or lower, according to the density program described on the horizontal axis in Fig. 3. Likewise, one of the ointment base ingredients, petrolatum, elutes between 0.35 and 0.55 g/mL consistently. Similarly, using the same chromatographic conditions as in Fig. 15, one can identify some of the major ingredients in a quencher lipstick formulation, as shown in Fig. 16. We believe that this method has considerable utility in deformulating products, particularly when lipophilic ingredients are present in sample matrix that can be solubilized ultimately in the SC–$CO_2$ mobile phase.

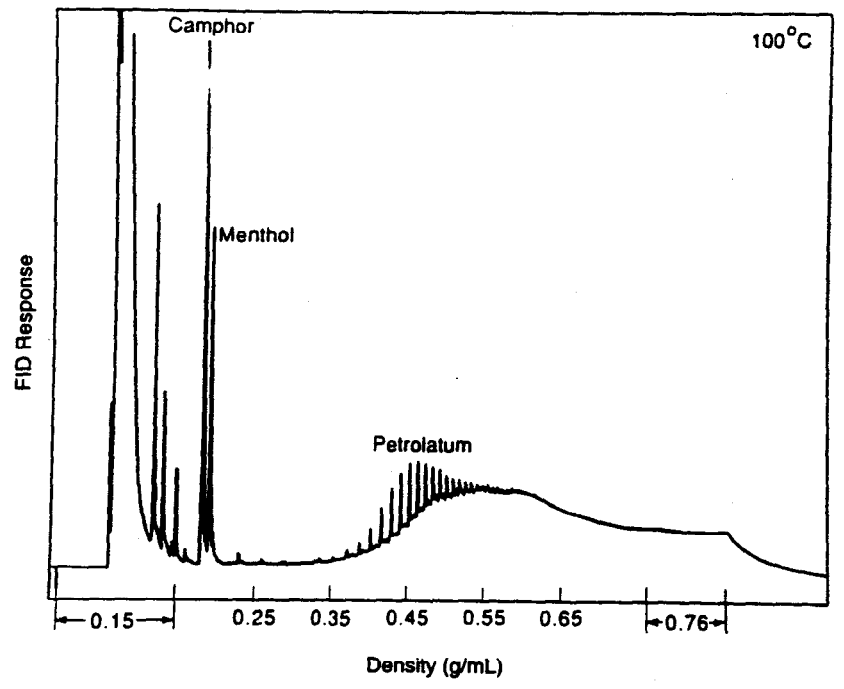


**FIG. 15.** Capillary SFC of lipophilic ingredients found in a decongestant ointment.

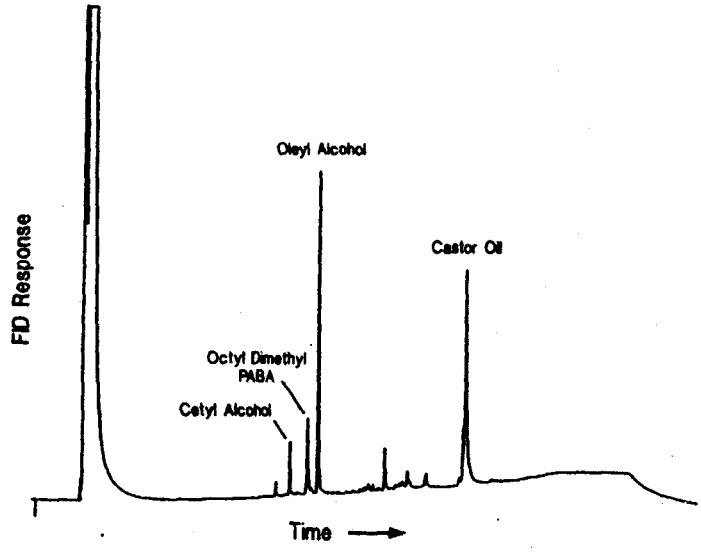


**FIG. 16.** Capillary SFC of components in quencher lipstick formulation.

### *Monitoring of Reactions*

The relative ease at which SFC can analyze lipid-containing matrices with little or no prior sample preparation has proven invaluable when monitoring reactions of interest in our research program. Others, such as Staby et al. (26), have applied SFC to the analysis of the reaction products from the transesterification of fish oils and noted the superiority of SFC over other means of analysis. In our case, the separation of mixtures of fatty acids (FA), fatty acid methyl esters (FAMEs), monoglycerides (MG), diglycerides (DG), and triglycerides (TG) by SFC has been utilized to determine the conversion of TGs to FAMEs (27). For example, Fig. 17 shows the conversion of soybean oil triglycerides to the corresponding FAMEs, as catalyzed by a supported lipase in the presence of flowing SC–$CO_2$, which serves as a reaction medium because it contains dissolved soybean oil and methyl alcohol. The SFC chromatogram in Fig. 17 shows that over a 97% conversion of the oil to FAMEs has been achieved. The capillary SFC separation in Fig. 17 was accomplished using an SB-Octyl-50 column (100 μm i.d., 10 m long, 0.5 μm film thickness) programmed from 120 atm (5 min) to 300 atm at a rate of 8 atm/min and a temperature gradient from 100°C (5 min) to 190°C at a rate of 8°C/min. Similarly, capillary SFC has been used to measure completeness of reaction during the development of an SFE/lipase-catalyzed reaction (SFE/SFR) method, used for the determination of fat food matrices (28).

Capillary SFC separation has also been achieved on the reaction products resulting from the enzymatically-catalyzed tranesterification of soybean oil with ethylene glycol to produce a mixture of mono- and diesters, which can be used in the lubri-

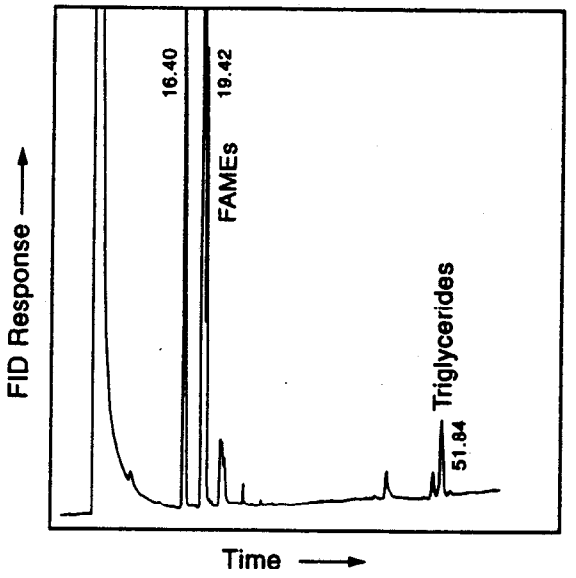


**FIG. 17.** Separation of FAMEs from soybean oil triglycerides using capillary SFC.

cation and the cosmetic industries. The reaction products, shown in Fig. 18, were produced by using a lipase (Novozyme 435, Novo Nordisk, Danbury, CT) in conjunction with SC–$CO_2$ to solubilize the starting reactants at 4000 psi (27.6 MPa) and 70°C, eventually yielding a product with 54.9% monoester and 16.7% diester content. Note that capillary SFC analysis also allows separation of the starting material (soybean oil TAG) and intermediate species such as di- and monoglycerides.

Similarly, SFC profiles can be generated on a native beeswax before and after it is enzymatically-transesterified, as shown in Fig. 19. In Fig. 19A, which is the capillary SFC of the native beeswax, the first cluster of peaks represents the odd-carbon number alkanes, $C_{25}$–$C_{33}$ , whereas the second cluster of peaks comprises the monoesters of palmitic acid with $C_{24}$–$C_{34}$ diols. The third cluster of peaks represents the diesters formed between the $C_{24}$–$C_{34}$ diols and palmitic acid, which elute last, based on their corresponding higher molecular weights. Transesterification with methyl acetate randomizes the alcohol chains, producing methyl esters and acetates

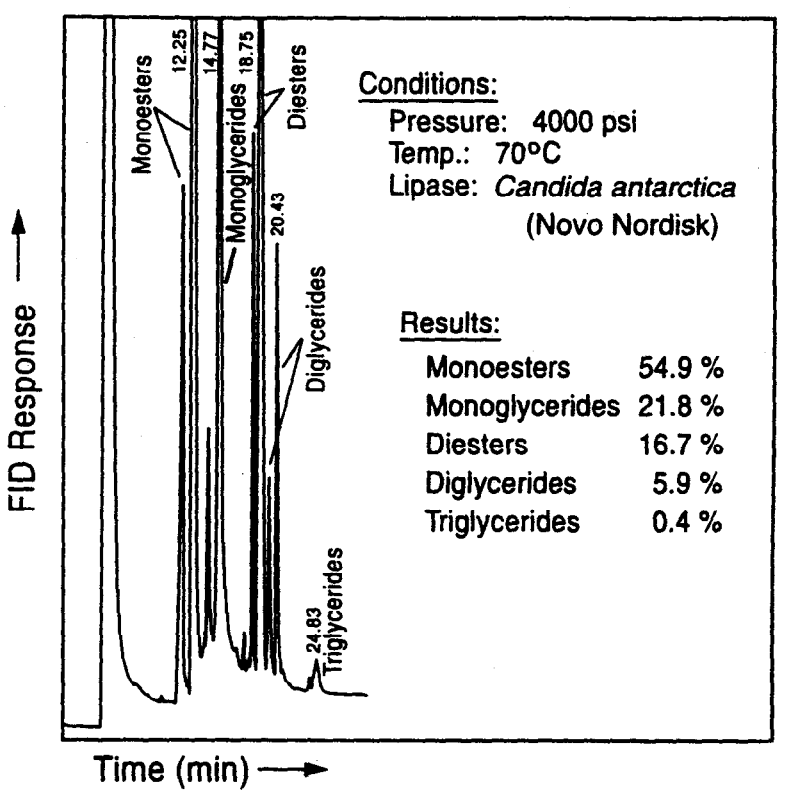


**FIG. 18.** Capillary SFC analysis of the product mixture obtained from the enzymatically catalyzed transesterification of soybean oil with ethylene glycol.

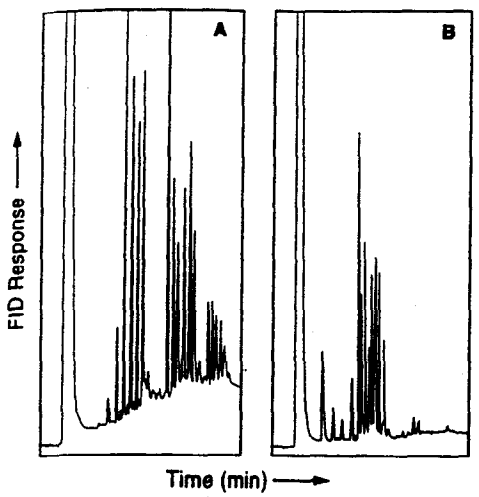


**FIG. 19.** Beeswax profile by capillary SFC(A) before and (B) after transesterification over an enzyme in flowing SC–$CO_2$.

of alcohols of lower molecular weight, leading to products having shorter retention times during the SFC run (Fig. 19B). Some of these aforementioned compounds, however, do overlap with the unreactive *n*-alkane cluster of peaks that were in the starting, native beeswax.

Another reaction sequence that we have studied by capillary SFC is a glycerolysis reaction run in the presence of SC–$CO_2$ in a stirred reactor (29). The SC–$CO_2$ in this case is apparently acting as an autocatalyst to initiate the production of mono- and diglycerides in the presence of low levels of water. The conditions for the SFC analysis were the same as the ones used for the aforementioned tranesterification reaction studies. Table 4 shows product compositions for the glycerolysis of five different vegetable oils from capillary SFC analysis. Note that free fatty acids are also detected by the capillary SFC runs, indicating that the competing hydrolysis reaction is also present in the stirred autoclave. Such data shows that glycerolysis conducted in an SC–$CO_2$ atmosphere can eliminate the use of alkali salts, traditionally used as glycerolysis catalysts, thereby avoiding the need to separate the catalyst from the final product.

**TABLE 4**
Composition (wt%) of Glycerolysis Products from Different Vegetable Oils[a]

| Type of Oil | MG[b] | DG[b] | TG[b] | FFA[b] |
|---|---|---|---|---|
| Soybean | 49.2 | 26.6 | 10.1 | 14.0 |
| Peanut | 46.6 | 32.1 | 12.5 | 8.8 |
| Cottonseed | 41.1 | 35.0 | 12.6 | 11.3 |
| Corn | 45.6 | 32.3 | 13.0 | 9.2 |
| Canola | 41.7 | 33.0 | 16.0 | 9.3 |

[a]Obtained at 250°C, 20.7 MPa, glycerol/oil ratio of 25, and addition of 4% water, 4-h reaction time.

[b]MG = monoglyceride; DG = diglyceride; TG = triglyceride; FFA = free fatty acid.

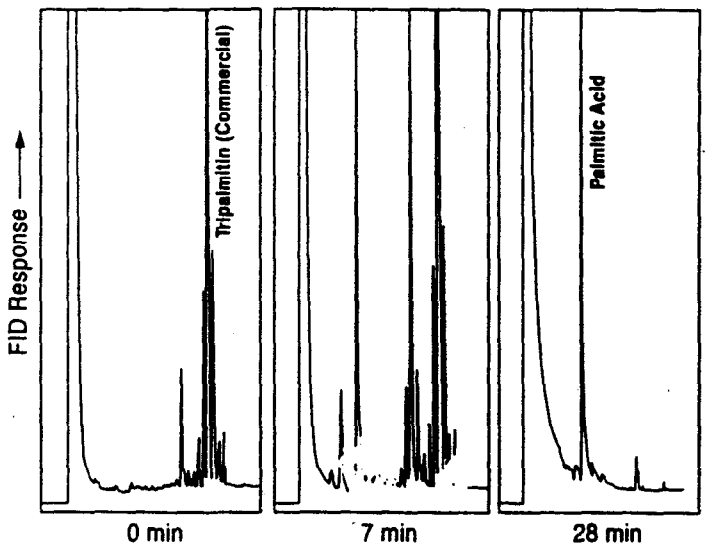


**FIG. 20.** Hydrolysis of tripalmitin in subcritical water as monitored by capillary SFC.

More recently, we have used capillary SFC to monitor the results from vegetable oil hydrolysis, conducted in subcritical water media to produce mixtures of fatty acids (30). The reaction temperature in this case is usually in the region of 250 to 300°C. As shown by the SFC trace (Fig. 20), a commercial tripalmitin sample can be converted to an intermediate series of diglycerides and palmitic acid after 7 min reaction time. SFC analysis also showed that total conversion to the acid could be achieved in 28 min (30). Although the resultant product mixtures had to be separated from an aqueous emulsion by solvent partition, SFC could be directly applied to the organic solvent layer for the assay of the lipophilic reaction products. The SFC analysis was accomplished on an SB-Phenyl-50 capillary column (50 μm i.d., 10 m long, 0.25 μm film thickness), using a pressure program that consisted of holding the pressure at 100 atm for 5 min, followed by a pressure ramp of 4 atm/min to 240 atm, and finally a ramp to 320 atm at a rate of 10 atm/min. The oven temperature was held isothermally at 100°C.

## Conclusions

In summary, we believe we have shown that SFC can be a vital technique for both basic and applied research, as illustrated by the numerous examples cited in this chapter. We have noted in this chapter only results obtained via capillary SFC using SC–$CO_2$ as a mobile phase with detection by FID. However, by using capillary SFC with modifiers (31) or other supercritical fluids, as well as packed column technology along with other supercritical fluid media, the SFC of very polar lipid species

can probably be achieved. Many of the separations noted in this chapter were accomplished in less than one hour of analysis time, suggesting that further optimization of the analysis time for specific target analytes may make these methods amenable for on-line process control. It should be noted that SFC is claiming new niche applications, particularly in the chromatography of chiral compounds and operation on a preparative scale.

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